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Homocysteine, *S*-adenosylmethionine and *S*-adenosylhomocysteine are associated with retinal microvascular abnormalities: the Hoorn Study

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ABSTRACT

The aim of the present study was to investigate the relationship between homocysteine and homocysteine metabolism components and retinal microvascular disorders in subjects with and without Type 2 diabetes. In this population-based study of 256 participants, aged 60–85 years, we determined total plasma homocysteine, SAM (*S*-adenosylmethionine) and SAH (*S*-adenosylhomocysteine) in plasma and erythrocytes, total folate in serum and erythrocytes, 5-MTHF (5-methyltetrahydrofolate), and vitamins B12 and B6. Participants were examined ophthalmologically by means of indirect funduscopy and two-field 45° fundus photography, and were graded for retinopathy and retinal sclerotic vessel abnormalities. A computer-assisted method was used to measure retinal vessel diameters. Total plasma homocysteine was inversely associated with retinal arteriolar diameters {standardized β, -0.20 [95% CI (confidence interval), -0.33 to -0.07]} or a decrease of 3.78 μm CRAEs (central retinal arteriolar equivalents) per 1 S.D. increase in homocysteine level ($=4.6$ μmol/l). In addition, the SAM/SAH ratio in plasma was inversely associated with retinal sclerotic vessel abnormalities and retinopathy [odds ratios, 0.61 (95% CI, 0.39–0.96) and 0.50 (95% CI, 0.30–0.83) per 1 S.D. respectively]. The associations were independent of age, sex, glucose tolerance status, other homocysteine metabolism components and cardiovascular risk factors. In conclusion, the results of the present study support the concept that total plasma homocysteine and a low SAM/SAH ratio in plasma, which may reflect reduced transmethylation reactions, may contribute to the pathogenesis of (retinal) microangiopathy.

Key words: *S*-adenosylmethionine, *S*-adenosylhomocysteine, homocysteine, methionine metabolism, retinal microvascular disease, retinopathy.

Abbreviations: AVR, arteriole-to-venule ratio; BMI, body mass index; CI, confidence interval; CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; CV, coefficient of variation; CVD, cardiovascular disease; DBP, diastolic blood pressure; HbA_{1c}, glycated haemoglobin; HDL-cholesterol, high-density lipoprotein cholesterol; IGM, impaired glucose metabolism; LDL-cholesterol, low-density lipoprotein cholesterol; 5-MTHF, 5-methyltetrahydrofolate; NGM, normal glucose metabolism; OGTT, oral glucose tolerance test; OR, odds ratio; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; SBP, systolic blood pressure.

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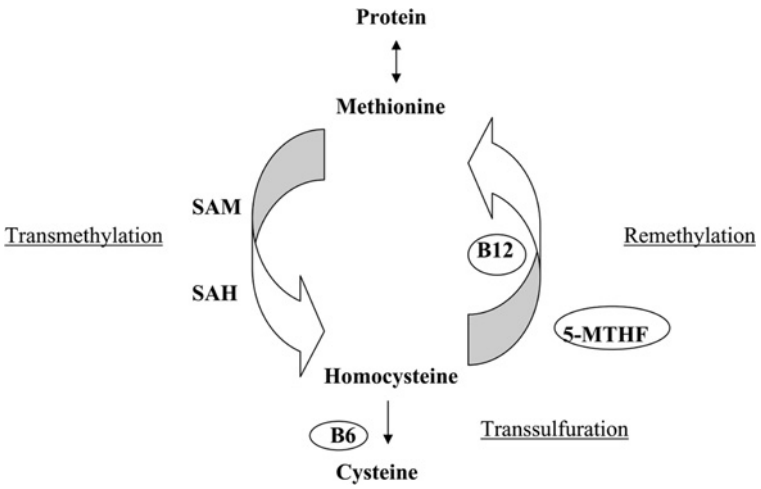

Figure 1 Methionine/homocysteine metabolism
B6, vitamin B6; B12, vitamin B12.

INTRODUCTION

Hyperhomocysteinaemia has been associated with an increased risk of cardiovascular disease independently of conventional cardiovascular risk factors [1,2]. It remains unclear, however, whether homocysteine itself is a direct cause of atherosclerosis. Other components of homocysteine metabolism, such as SAM (*S*-adenosylmethionine), SAH (*S*-adenosylhomocysteine) and folate, have also been suggested to play a role in, and possibly mediate, the relationship with cardiovascular disease [3–6]. Methionine/homocysteine metabolism involves methionine transmethylation, which involves the formation of SAM and SAH, and leads to the production of homocysteine (Figure 1). Homocysteine can be remethylated to methionine, which requires vitamin B12 and folate, or can go through the trans-sulfuration pathway, which requires vitamin B6. However, the exact extent of contribution of these metabolites to cardiovascular disease risk has not yet been established [7].

Homocysteine is thought to increase cardiovascular risk by inducing endothelial dysfunction, increasing oxidative stress, and inducing thrombophilia and proliferation of smooth muscle cells, which can lead to narrowing of the intravascular lumen [2,7–9]. Experimental studies, mainly in rats, suggest that hyperhomocysteinaemia may also play a role in the pathogenesis of renal microvascular disease [10–12]. In humans, however, little is known about the relationship between homocysteine and its metabolites and microangiopathy. High levels of homocysteine were reported to be strongly associated with the development of microalbuminuria [13,14]. Hyperhomocysteinaemia is also thought to be a strong risk factor for retinal vascular occlusive disease [15]. For retinopathy, this relationship is not very clear. In the Hoorn study, we have reported previously a relationship between hyperhomocysteinaemia and retinopathy in individuals with Type 2 diabetes [16], whereas others did not find any relationship [17].

In light of these considerations, we hypothesized that homocysteine and (or) components of homocysteine metabolism are involved in the pathogenesis of microvascular disease. As the retina offers a unique opportunity to non-invasively analyse the microvasculature, we investigated the relationship between homocysteine and components of homocysteine metabolism and computer-assisted measurements of retinal vessel diameters, retinal sclerotic vessel abnormalities and retinopathy. Because hyperhomocysteinaemia appears to be more strongly related to cardiovascular disease in the presence of Type 2 diabetes [18], we studied the relationships mentioned above in a population-based study of subjects with and without Type 2 diabetes.

MATERIALS AND METHODS

Study population

For the present study, we used data from the 2000–2001 Hoorn Study follow-up examination. The Hoorn Study is a population-based cohort study of Type 2 diabetes and its cardiovascular complications among 2484 Caucasian subjects, aged 50–74 years, which started in 1989. Full details have been provided elsewhere [19,20]. Fasting and 2-h post-load plasma glucose levels after a 75-g OGTT (oral glucose tolerance test) were measured in plasma, and were used for classification into glucose tolerance categories [21]. Subjects who were already known to have diabetes or were using glucose-lowering treatment

were excluded from the OGTT. At baseline, an age-, sex- and glucose-tolerance-stratified subsample of 631 participants was studied extensively for reasons of efficiency. In 2000–2001, a follow-up examination was carried out among surviving participants who gave their permission to be re-contacted [20]. Of the 631 participants who had an ophthalmological examination at baseline, approx. 60 % dropped out of the follow-up examination because of the following reasons: 119 (19 %) had died, 49 (8 %) had moved out of the region and 207 (33 %) did not participate because of mobility or health problems, or lack of motivation. Finally, 256 subjects were included in the follow-up ophthalmological examination [22], 70 with NGM (normal glucose metabolism), 69 with IGM (impaired glucose metabolism) and 109 with Type 2 diabetes [21].

Written informed consent was obtained from all participants. The Ethical Review Committee of the VU University Medical Center (VUmc), Amsterdam, The Netherlands, approved the Hoorn Study.

Sample preparation and determination of homocysteine, SAM, SAH, folates and vitamins

All samples were processed within 30 min after collection, stored at $-80\,^{\circ}C$ and analysed within 2 years. EDTA-anticoagulated blood samples were put on ice for the determination of homocysteine, SAM and SAH. For SAM and SAH measurements, samples were deproteinized immediately by the addition of 0.625 ml of a 10 % perchloric acid solution to 1 ml of plasma and by adding 1 ml of 5 % perchloric acid to 1 ml of whole blood, followed by mixing [23]. For the determination of total folate, 0.5 mg of ascorbic acid was added to 0.5 ml of serum [23] and, for the determination of total folate in erythrocytes, 1 ml of reagent with ascorbic acid, human serum albumin and sodium azide (Chiron Diagnostics) was added to 50 μl of whole blood.

Total plasma homocysteine was determined with an automated fluorescence polarization immunoassay on an Abbott Imx analyser {interassay CV (coefficient of variation) was 4 %, where CV = S.D. of the mean difference/[(square root of 2) × pooled mean]} [24]. Tandem MS was used to determine SAM and SAH in plasma and whole blood (intra-assay CVs were 4 % for both determinations, and inter-assay CVs were 8 % and 6 % respectively) [25]. Erythrocyte concentrations of SAM and SAH were calculated as: [(whole blood concentration−plasma concentration) × (1−haematocrit)]/haematocrit. Total folate in serum and erythrocytes and serum vitamin B12 were measured with automated chemiluminescence (180® Automated Chemiluminescence Systems; Chiron Diagnostics). Intra- and inter-assays CV were 4 % and 5 % for total folate respectively. 5-MTHF (5-methyltetrahydrofolate), the active form of folate, was measured by HPLC [26], which was also used to measure plasma vitamin B6 [27]. The inter-assay CV was 7 %.

Ophthalmological examination and retinal vessel diameter measurements

After mydriasis with 0.5 % tropicamide and 2.5 % phenylephrine eye drops, the retina was examined by funduscopy and fundus photography, as described previously [22]. Briefly, fundus photography was performed with a 45 °CR5 non-mydriatic retinal camera (Canon), interfaced to a 3CCD colour video camera (Sony). The quality of each photograph was checked immediately on a colour video monitor (Trinitron; Sony) and a new photograph was taken if the quality was insufficient. The photographs were digitized, compressed and stored on a magneto-optical disc using the TEAC MV-300P Viewfile system. Two photographs were made of each eye, one centred on the macula and one centred on the optic disc.

Methods used to measure retinal vessel diameters from digitized photographs followed a standardized protocol, which has been described elsewhere [28]. Briefly, one investigator, blinded to the participant identity, independently measured all arterioles and venules 0.5 to 1 disc diameter from the optic disc margin using a computer-imaging program (Retinal Analysis; Optimate). The branches of arterioles were also measured if the trunk measures were $\geqslant 85$ μm. Computer-assisted measurements of the diameters of arterioles and venules were obtained and combined according to the revised formulas of Parr and Spears [29,30] and Hubbard et al. [28,31], which account for magnification differences and the number of vessels in photographs. Average diameters of arterioles [CRAEs (central retinal arteriolar equivalents)] and venules [CRVEs (central retinal venular equivalents)] in one eye were assessed, and combined into an AVR (arteriole-to-venule ratio). An AVR of 1.0 indicates that the diameters of the arterioles are approximately equal to the diameters of the venules, whereas a smaller AVR indicates narrower arterioles or wider venules. For each subject, one photograph centred on the optic disc was used, alternately selected from the left and right eye (ratio, 50:50). In the case of insufficient quality, the photograph of the other eye was examined. The intra-observer intersession CVs were 5 % for CRVEs, 8 % for CRAEs and 9 % for AVRs.

Both fundus photographs were independently analysed by two individuals to grade retinal sclerotic vessel abnormalities and retinopathy. In the case of disagreement, the judgment of a third investigator was taken to be decisive. Retinal sclerotic vessel abnormalities were defined as the presence of venous beading, focal narrowing, arteriovenous crossing changes, 'copper' or 'silver' wiring, dilated or tortuous retinal veins, or central or branch venular occlusion. Retinopathy was

defined as the presence of one or more microaneurysms, haemorrhages or hard exudates, possibly in combination with areas of neovascularization, fibrous proliferation, pre-retinal or vitreous haemorrhages and/or laser coagulation scars in at least one eye according to the Eurodiab classification [22,32].

Other measurements

Brachial SBP (systolic blood pressure) and DBP (diastolic blood pressure), HbA_{1c} (glycated haemoglobin), fasting insulin, serum total, HDL-cholesterol (high-density cholesterol), LDL-cholesterol (low-density lipoprotein cholesterol), serum triacylglycerols (triglycerides), serum creatinine, serum albumin, BMI (body mass index), waist and hip circumferences, smoking and prior CVD (cardiovascular disease) were determined according to methods described elsewhere [33–35]. Hypertension was defined as a DBP $\geqslant 90$ mmHg, a SBP $\geqslant 140$ mmHg and/or the use of antihypertensive medication [36].

Statistical analyses

Clinical and ophthalmological characteristics, expressed as means $\pm$ S.D., percentages or medians (interquartile range), in the case of a skewed distribution, were computed according to tertiles of total plasma homocysteine levels. Overall group differences in continuous variables were tested by means of ANOVA, and the differences in categorical measures were tested with Pearson's χ^2 test.

Multivariable linear regression analyses were used to calculate the associations between homocysteine and its metabolites and AVRs, CRAEs and CRVEs. To make the results of linear regression models comparable among different determinants, standardized β values are reported. A standardized β value of 0.1 indicates that, if the determinant increases by 1 S.D., the outcome increases by 0.1 S.D. Furthermore, changes in AVRs, CRAEs and CRVEs are reported. Logistic regression analyses were used to calculate the associations of components of homocysteine metabolism with retinal sclerotic vessel abnormalities and retinopathy [ORs (odds ratios) are reported per 1 S.D.]. Because all of the determinants had linearity with AVRs, CRAEs, CRVEs, retinal sclerotic vessel abnormalities and retinopathy, homocysteine and its metabolism components were used as continuous variables in the models. First, we adjusted the associations for the stratification variables age, sex and glucose tolerance status. Then, we additionally adjusted the associations of the other components of homocysteine metabolism and for other potential confounders, i.e. HbA_{1c}, SBP, DBP, BMI, total cholesterol, microalbuminuria, and prior CVD.

Effect-modification by Type 2 diabetes was investigated by entering product terms of diabetes (yes/no) $\times$ the predictor variable in the regression models. P values < 0.05 were considered statistically significant. All analyses were performed in SPSS 12.0 for Windows 98.

RESULTS

Of the 256 participants, six had photographs that could not be graded for AVRs and CRAEs, and two had photographs that could not be graded for CRVEs. Furthermore, four subjects had data missing for total plasma homocysteine, and 68 subjects had data missing for one or more components of the homocysteine metabolism. Subjects with one or more missing variables did not differ substantially from subjects with available data (results not shown) and, therefore, were not excluded from further analyses.

The CRAE was $170.6 \pm 18.9\ \mu$m (range, 119.9–225.1 μm), CRVE was $231.2 \pm 27.1\ \mu$m (range, 148.7–333.5 μm) and AVR was $0.75 \pm 0.10\ \mu$m (range, 0.49–1.31 μm). Of the 256 subjects, 31 had retinal sclerotic vessel abnormalities and 32 had retinopathy.

Clinical characteristics

Table 1 shows the clinical and ophthalmological characteristics according to the tertiles of total plasma homocysteine levels. AVRs and CRAEs were inversely correlated with levels of homocysteine.

Associations between homocysteine, SAM and SAH and retinal vessel diameter

Figure 2(a) shows the distribution of homocysteine levels against CRAEs for subjects with NGM, IGM and diabetes. Figure 2(b) shows that, after adjustment for age, sex and glucose tolerance status, subjects in the highest tertile of homocysteine had CRAEs 8 μm lower than subjects with homocysteine levels in the lowest tertile ($P < 0.05$). Indeed, when homocysteine was analysed as a continuous variable, total plasma homocysteine was inversely associated with CRAEs and AVRs after adjustment for age, sex and glucose tolerance status (Table 2). This implies that the CRAE decrease of 3.78 μm per 1 S.D. increase in homocysteine level ($=4.6\ \mu$mol/l) (Table 2). After additional adjustments for plasma SAM, 5-MTHF and cardiovascular risk factors, the association of total plasma homocysteine with small retinal arteriolar diameter did not change (Table 3). Furthermore, individual adjustment for other components of homocysteine metabolism and other cardiovascular risk factors, such as lipid levels or current smoking, also did not affect the results (results not shown).

High plasma SAM and SAH were both associated with lower AVRs after adjustment for age, sex and glucose tolerance status (Table 2). After adjustment for 5-MTHF, plasma homocysteine, plasma SAM, plasma SAH, BMI, waist-to-hip ratio, waist circumference or microalbuminuria, the associations lost statistical significance. After adjustment for total folate in serum and erythrocytes, or vitamins B6 and B12, the association between SAM and SAH in plasma and lower AVRs remained statistical significant (results not shown).

Table 1 Baseline characteristics according to the tertiles of homocysteine levels

Values are means ± S.D. or percentages, or medians (interquartile range) in the case of skewed distribution. *P* values were calculated by ANOVA or a χ^2 test.

	Homocysteine tertile			
	< 10.0 μmol/l	10.0–12.7 μmol/l	⩾ 12.8 μmol/l	*P* value trend
n	85	83	84	
Age (years)	69 ± 6	72 ± 7	74 ± 7	< 0.001
Gender (% male)	47	49	61	0.076
HbA_{1c} (%)	6.3 ± 1.0	6.3 ± 0.9	6.2 ± 0.9	0.262
Diabetes (%)	41	46	42	0.894
Hypertension (%)	75	77	82	0.351
SBP (mmHg)	144 ± 19	150 ± 21	148 ± 23	0.181
DBP (mmHg)	85 ± 11	85 ± 12	83 ± 12	0.344
Use of ACE-inhibitor/calcium antagonist (%)	18.8	21.7	21.4	0.730
BMI (kg/m^2)	27.2 ± 4.0	27.2 ± 3.6	27.8 ± 4.5	0.345
Waist-to-hip ratio	0.94 ± 0.10	0.94 ± 0.09	0.95 ± 0.09	0.306
Waist circumference (cm)	95.6 ± 12.7	95.5 ± 11.4	97.9 ± 10.4	0.200
Total cholesterol (mmol/l)	5.7 ± 1.1	5.9 ± 1.0	5.6 ± 1.0	0.623
HDL-cholesterol (mmol/l)	1.3 ± 0.4	1.5 ± 0.4	1.3 ± 0.4	0.862
LDL-cholesterol (mmol/l)	3.5 ± 0.9	3.7 ± 0.9	3.6 ± 0.8	0.861
Triacylglycerols (mmol/l)	1.5 (1.0–2.0)	1.4 (1.0–1.8)	1.4 (1.1–1.7)	0.186
Statin therapy (%)	17.6	12.0	10.7	0.375
Microalbuminuria (%)	14	19	21	0.234
Albumin-to-creatinine ratio	0.7 (0.5–1.3)	0.7 (0.5–1.6)	0.8 (0.5–1.9)	0.037
Current smoking (%)	11	10	16	0.329
Prior CVD (%)	45	59	57	0.261
AVR	0.76 ± 0.12	0.75 ± 0.09	0.72 ± 0.10	0.024
CRAE (μm)	173 ± 19	172 ± 18	166 ± 19	0.011
CRVE (μm)	231 ± 30	232 ± 27	231 ± 25	0.900
Retinal vessel sclerosis (%)	9	18	17	0.184
Retinopathy (%)	9	16	13	0.458

Associations between SAM and SAH and retinal sclerotic vessel abnormalities and retinopathy

The SAM/SAH ratio in plasma was lower in subjects with retinal sclerotic vessel abnormalities than in subjects without retinal sclerotic vessel abnormalities [5.6 (4.5–6.5) compared with 6.0 (5.1–7.0) respectively; $P = 0.05$]. In addition, plasma SAH was significantly higher and the plasma SAM/SAH ratio was significantly lower in subjects with retinopathy compared with subjects without retinopathy [plasma SAH, 17.6 (15.6–25.6) compared with 15.3 (12.5–20.2) nmol/l respectively ($P = 0.003$); plasma SAM/SAH ratio, 5.1 (4.2–5.8) compared with 6.0 (5.1–7.0) respectively ($P < 0.001$)].

After adjustment for age, sex and glucose tolerance status, the plasma SAM/SAH ratio was inversely and strongly associated with retinal sclerotic vessel abnormalities and retinopathy (Table 2). Adjustments for other homocysteine metabolism components had little impact on either association. Further adjustments for cardiovascular risk factors did not change the results substantially, except for prior CVD, which slightly attenuated the association of retinal sclerotic vessel abnormalities (results not shown).

In addition, the plasma SAM/SAH ratio had a borderline association with microalbuminuria after adjustment for age, sex and glucose tolerance status {microalbuminuria (yes/no) OR, 0.81 [95% CI (confidence interval), 0.62–1.06]}. The standardized β value for microalbuminuria as a linear variable was −0.153 ($P = 0.029$; S.D. = 28.1) (results not shown).

Additional analyses

The presence of Type 2 diabetes did not statistically significantly ($P > 0.05$) modify the associations between homocysteine metabolism components and AVRs, CRAEs, CRVEs, retinal sclerotic vessel abnormalities or retinopathy. Erythrocyte SAM and SAH, the erythrocyte SAM/SAH ratio, 5-MTHF, total folate in serum and in erythrocytes, and vitamins B12 and B6 were not associated with AVRs, CRAEs, CRVEs, retinal sclerotic vessel abnormalities and retinopathy (results not shown).

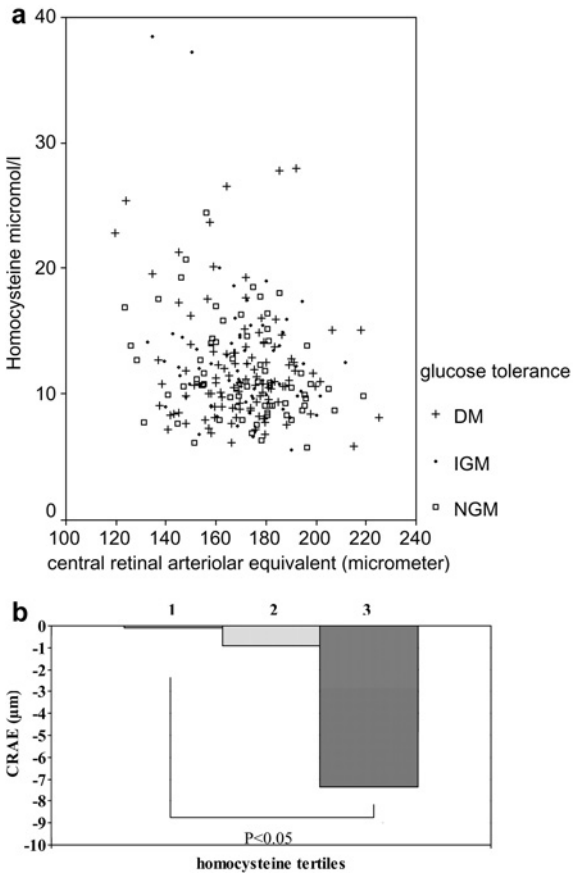

Figure 2 CRAEs and homocysteine in the study subjects (a) and in the homocysteine tertiles (b)

(a) CRAEs plotted against homocysteine levels in subjects with NGM, IGM and diabetes (DM). (b) Age-, sex- and glucose-tolerance-status-adjusted regression coefficients of tertiles of total plasma homocysteine associated with retinal arteriolar diameter. Homocysteine tertiles: 1, <10.0 μmol/l; 2, 10.0–12.7 μmol/l; and 3, $\geqslant 12.8$ μmol/l.

DISCUSSION

The present study, which included subjects without and with Type 2 diabetes, had two main findings. First, a high total plasma homocysteine level was significantly associated with retinal arteriolar narrowing, independently of other components of homocysteine metabolism or cardiovascular risk factors. Secondly, a lower plasma SAM/SAH ratio was independently associated with retinal sclerotic vessel abnormalities and retinopathy. These results did not differ between subjects with or without Type 2 diabetes.

The present study is the first to show an independent association between total plasma homocysteine and retinal arteriolar narrowing. This finding is in line with the general concept that homocysteine has vasculotoxic properties. Homocysteine is considered an independent risk factor for atherosclerotic vascular disease and

Table 2 Age-, sex- and glucose-tolerance-status-adjusted associations between homocysteine, components of homocysteine metabolism and retinal vascular abnormalities

Values are calculated with linear regression analyses (for AVR, CRAE and CRVE) or with logistic regression analyses (for RSVC and retinopathy) and are shown as standardized β values (St. β) (95 % CI)/change in AVR or μm CRAE/CRVE and ORs per 1 S.D. (95 % CI). RSVC, retinal sclerotic vessel abnormalities.

	S.D.	AVR [St.β (95 % CI)/change in ratio]	CRAE [St.β (95 % CI)/change in μm]	CRVE [St.β (95 % CI)/change in μm]	RSVC [OR (95 % CI)]	Retinopathy [OR (95 % CI)]
		0.1	18.9 μm	27.1 μm		
Homocysteine	4.6 μmol/l	−0.15 (−0.28 to −0.01)/−0.02	−0.20 (−0.33 to −0.07)/−3.78	−0.04 (−0.17 to 0.09)/−1.03	0.97 (0.65–1.45)	0.98 (0.65–1.47)
SAM-plasma	28.5 nmol/l	−0.13 (−0.26 to 0.00)/−0.01	−0.05 (−0.18 to 0.07)/−0.95	0.10 (−0.02 to 0.23)/2.71	0.92 (0.62–1.36)	0.94 (0.64–1.40)
SAH-plasma	9.2 nmol/l	−0.15 (−0.29 to −0.01)/−0.02	−0.12 (−0.25 to 0.01)/−2.27	0.06 (−0.07 to 0.19)/1.63	1.17 (0.85–1.62)	1.16 (0.84–1.60)
Plasma SAM/SAH ratio	1.5	0.05 (−0.10 to 0.20)/0.01	0.09 (−0.05 to 0.23)/1.70	0.02 (−0.12 to 0.16)/0.54	0.61 (0.39–0.96)	0.50 (0.30–0.83)
5-MTHF	8.9 nmol/l	0.02 (−0.13 to 0.16)/0.00	−0.00 (−0.14 to 0.13)/−0.07	0.00 (−0.14 to 0.14)/−0.11	0.98 (0.67–1.44)	0.92 (0.59–1.45)
Vitamin B12	101.1 pmol/l	−0.01 (−0.14 to 0.13)/−0.00	−0.03 (−0.16 to 0.09)/−0.57	−0.02 (−0.15 to 0.12)/−0.54	1.11 (0.74–1.64)	1.01 (0.68–1.49)
Vitamin B6	37.2 nmol/l	0.03 (−0.10 to 0.16)/0.00	−0.06 (−0.18 to 0.06)/−1.13	−0.10 (−0.22 to 0.03)/−2.71	1.30 (0.97–1.74)	1.01 (0.70–1.45)

Table 3 Multivariate associations of homocysteine with CRAEs

Results are calculated with linear regression analyses, and are shown as standardized β values (St. β) (95 % CI)/change in μm.

Model	Added variables	CRAE [St.β (95 % CI)/change in μm]
1	Homocysteine, age, sex and glucose tolerance status	− 0.20 (− 0.33 to − 0.07)/− 3.78
2	As model 1+plasma SAM and 5-MTHF	− 0.25 (− 0.39 to − 0.09)/− 4.73
3	As model 1+HbA_{1c}, SBP, DBP, BMI, total cholesterol, microalbuminuria and prior CVD	− 0.22 (− 0.35 to − 0.09)/− 4.16
4	As model 2+HbA_{1c}, SBP, DBP, BMI, total cholesterol, microalbuminuria and prior CVD	− 0.25 (− 0.41 to − 0.09)/− 4.73

mortality [1,2]. In addition, hyperhomocysteinaemia has been associated with retinal vascular occlusive disease, which was shown recently in a meta-analysis [15]. In contrast with our results, one study including 84 recently diagnosed Type 2 diabetic individuals and 115 non-diabetic individuals did not find a relationship between plasma homocysteine levels and AVR [37]. That study, however, did not use a computer-assisted method to accurately measure arteriolar and venular diameters, as we have done in the present study. Further support for an association between hyperhomocysteinaemia and microvascular disease derives from studies of renal microvascular disease [11,13,14], which show an independent association of homocysteine with the presence and development of microalbuminuria [13,14]. In addition, Fassbender et al. [38] demonstrated that homocysteine levels were increased in 82 subjects with cerebral microangiopathy compared with subjects without cerebrovascular disease. Therefore our present study and previous studies suggest that hyperhomocysteinaemia may not only be a risk factor for macrovascular disease, but may also be associated with microvascular disease in the retina and elsewhere.

Our present results have shown that subjects with homocysteine levels in the highest tertile had an 8 μm smaller retinal arteriolar diameter compared with subjects with homocysteine levels in the lowest tertile. To put this into perspective, higher blood pressure (a known determinant of retinal arteriolar narrowing) was, in our present study, associated with decreases of 0.6 and 1.1 μm in retinal arteriolar diameter per 10 mmHg higher SBP and DBP respectively, which is comparable with previous findings [39,40]. Therefore these findings suggest that homocysteine could be a biologically relevant risk factor for retinal arteriolar narrowing.

The exact mechanism by which homocysteine affects the vascular system is not completely understood. However, homocysteine is thought to exert its toxic effects on the vascular wall by impairing endothelial function, increasing oxidative stress, decreasing availability of NO, inducing a prothrombotic state and inducing proliferation of smooth muscle cells [2,9]. This may cause vasoconstriction, intimal thickening, medial hyperplasia, hyalinization and sclerosis, consequently leading to a smaller arteriolar lumen.

We did not find any association between homocysteine and prevalent retinopathy. Previously, Hoogeveen et al. [16] found a significant relationship between homocysteine and retinopathy in diabetic subjects, but not in non-diabetic subjects in the Hoorn Study population in 1989. These discordant findings might be due to the limited power of the present study ($n = 32$ with retinopathy), which might also explain the absence of an interaction with diabetic status. Moreover, selective mortality of diabetic individuals with hyperhomocysteinaemia [41] may have resulted in a 'healthy survivor effect' and may have affected our results. On the other hand, results on the association between homocysteine and diabetic retinopathy are not consistent [42,43].

Unlike total plasma homocysteine, a lower plasma SAM/SAH ratio was strongly associated with retinal sclerotic vessel abnormalities and retinopathy. Previous studies have shown that a low SAM/SAH ratio was associated with end-stage renal failure [44], peripheral arterial occlusive disease [23] and vascular disease (stroke and myocardial infarction) [45]. Our additional results that the plasma SAM/SAH ratio was associated, in a borderline significant manner, with microalbuminuria support these findings. Indeed, the ratio of SAM and SAH is crucial in the regulation of multiple enzymatic transmethylation reactions [46]. A decrease in this ratio may result in inhibition of transmethylation reactions, which affects the biosynthesis of proteins, hormones, phospholipids, neurotransmitters, RNA and DNA [47,48] and, consequently, might lead to inhibition of vascular endothelial cell growth [49] and impairment of endothelial function. In addition, increased SAH, which could be caused by elevated homocysteine concentrations, also potentially acts as an inhibitor of transmethylation reactions [46]. Taken together, we hypothesize that a decrease in transmethylation reactions may be an important factor in the pathogenesis of retinal microvascular disease.

In the present study, we used a detailed measurement of retinal microvessel diameters by a computer-assisted method on fundus photographs made in mydriasis. Moreover, we used the revised formulas of Parr and Hubbard to quantify vessel calibre, by which the vessels were measured independently of image scale and the number of measured vessels [31]. A second advantage

of the present study is its population-based design, including subjects with and without Type 2 diabetes.

The present study also has limitations though. The result that homocysteine is associated with retinal arteriolar narrowing and plasma SAM/SAH ratio is only associated with sclerotic vessel abnormalities and retinopathy might be confusing, suggesting different pathophysiological mechanisms. As the complete pathophysiological pathway of the association between homocysteine/methionine metabolism and microangiopathy is still unclear, our results may represent different pathophysiological pathways; however, we cannot exclude the possibility that these differences are caused by chance. Owing to the cross-sectional design of the present study, we cannot interpret the results as cause-and-effect relationships. In addition, the study population consisted of individuals aged 60–85 years with a considerable prevalence of cardiovascular risk factors, and we do not know whether the results of the present study can be generalized to a younger or healthier population, or to other ethnicities. However, an older population also has advantages in the study of homocysteine and its components, because homocysteine levels increase with age and low to low-normal concentrations or deficiencies in folate and vitamin B12 are relatively common in this age group, which provide wider ranges of concentrations to study in the elderly.

In conclusion, this is the first population-based study that shows an independent relationship of total plasma homocysteine with retinal arteriolar narrowing. In addition, the plasma SAM/SAH ratio was strongly associated with retinal sclerotic vessel abnormalities and retinopathy. This suggests that homocysteine and, possibly, reduced transmethylation reactions may be involved in the pathogenesis of retinal microvascular disease. Further studies are needed to clarify the microvascular effects of homocysteine-lowering treatment with folate, which may also increase transmethylation reactions [50], in order to create new treatment modalities for (retinal) microvascular disease.

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